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Thesis

AUTOLOGOUS MESENCHYMAL STEM CELLS IN NONUNION FRACTURES

by

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B.A., Grinnell College, 2013

Submitted in partial fulfillment of the

requirements for the degree of

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AUTOLOGOUS MESENCHYMAL STEM CELLS IN NONUNION FRACTURES

JOHN DREIER

ABSTRACT

The current gold standard of therapy for treatment of tibial fracture nonunion is iliac crest bone graft. However, this intervention is associated with significant morbidity to the donor site. Research into alternative interventions highlights the role of mesenchymal stem cells (MSCs). MSCs are capable of differentiating into mature, organized osseous tissue as well as recruiting local vascular cells. However, there are few prospective studies demonstrating the therapeutic potential of MSCs in fracture nonunion. The proposed study is a multicenter single-blinded controlled study of MSC application compared to iliac crest bone graft in the setting of fracture nonunion of the tibia. The study subjects will be evaluated at each return to care with mRUST radiographic scoring as well as Short-Form 12 evaluation of general health. These results will be correlated with MSC concentrations as assessed by FACS analysis. The data from this study will help to characterize MSCs as a possible therapeutic intervention in fracture nonunion.

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LIST OF ABBREVIATIONS

BMP ..... Bone Morphogenic Proteins

IL-1 ..... Interleukin 1

IL-6 ..... Interleukin 6

ISO ..... International Standards Organization

MCS ..... Mental Component Summary

mRUST ..... modified Radiographic Union of Tibia

MSC ..... Mesenchymal Stem Cell

NSAID ..... Non-steroidal anti-inflammatory drug

PCS ..... Physical Component Summary

PDGF ..... Platelet derived growth factor

PTH ..... Parathyroid Hormone

SF-12 ..... Short Form 12

TGF-β ..... Transforming growth factor β

TNFα ..... Tumor necrosis factor α

VEGF ..... Vascular endothelial growth factor

INTRODUCTION

Background

Fractures are the most common large-organ traumatic injuries in the general population, and osteoporotic fractures are the fastest growing health care problem of aging. In 2004, costs related to treating fractures approached $24 billion and projections estimate that costs of treating injuries from falls will reach $64 billion by 2020.[1] While the processes of fracture repair after surgery are usually optimal, the healing of up to 10% of the estimated ~8 to 10 million fractures that occur annually in the United States is delayed or impaired.[2] Due to repeated surgical intervention, disability of the affected location, and extended patient pain, patients with nonunion fractures require more healthcare services than patients who heal within the typical 4-6 weeks.[3] Nonunion patients face recovery times ranging from months to years while incurring medical costs and requiring strong opioids for chronic pain secondary to fracture.[3] Risk of nonunion is elevated in severe fractures, such as those that are open or have extensive comminution (multiple fracture fragments) on plain film, and those in locations with poor vascular supply, like the distal tibia or scaphoid.[4] Additional risk factors for fracture nonunion include high body mass index, smoking, diabetic condition and alcoholism (see Table 1).[4]

Table 1. Local and systemic factors predisposing and contributing to nonunion. Adapted with permission from Harwood 2010.[5]

Predisposing Factors		Contributing Factors
Mechanical Instability	Inadequate fixation	Infection
	Distraction	Smoking

	Bone loss	Certain medications
	Poor bone quality	Advanced age
Inadequate vascularity	Severe injury	Systemic medical conditions
	Excessive soft tissue stripping	Poor functional level
	Vascular injury	Venous stasis
Poor bone contact	Soft tissue interposition	Burns
	Malposition or malalignment	Radiation
	Bone loss	Obesity
	Distraction	Alcohol abuse
		Metabolic bone disease
		Malnutrition
		Vitamin deficiencies

The current gold standard of therapy for nonunion fractures involves the application of autologous iliac crest bone grafts. However, the associated morbidity of the graft harvest coupled with recent advances in our understanding of fracture biology has spurred research into alternative therapeutic options.[6] Biologics and small molecule therapies, such as bone morphogenic proteins (BMPs) and parathyroid hormone (PTH),[7] as well as device oriented therapeutics, such as platelet-rich plasma scaffolding,[8] ceramic implantation,[9] ultrasound delivery of osteogenic bioactive molecules,[10] and application of mesenchymal stem cells,[11] have been explored as possible avenues of therapy for fracture nonunion. Mesenchymal stem cell application in particular shows promise in early clinical trials.[12,13]

Mesenchymal stem cells are multipotent postnatal progenitor cells originally isolated from bone marrow.[14] Early experiments demonstrated that when these self-renewing cells are selectively cultured and transplanted in mice they form organoids, or organized osseous tissue containing hematopoietic marrow with sophisticated trabecular framework.[15] Thus, MSCs have inherent osteogenic potential and could be utilized therapeutically in bone modeling and repair. Additionally, *in vitro* studies have shown that MSCs can be induced to differentiate into multiple mesenchymal tissue types, including bone marrow stroma, cartilage, bone, adipose, muscle, tendons, and connective tissue.[16] However, contention remains as to whether the stem cell nature (i.e. self-renewal, multipotency) of the cell populations used in these experiments is verifiable. Indeed, a substantial portion of the literature treats MSCs as "medicinal signaling cells," or cells that influence the cellular microenvironment via paracrine factors rather than cells capable of regenerating tissue. With this schema, investigators have explored the purported immunomodulatory potential of MSCs in a variety of disease states and conditions ranging from myocardial infarction to inflammatory bowel disease.[17]

Statement of the Problem

Fracture nonunion is a severe, debilitating and costly condition. As the United States population ages, age-related fractures are expected to increase.[4] With an overall nonunion rate of 5 to 10 percent, while in some fractures sites and types and in conjunction with specific co-morbidities can approach a 50% nonunion rate, novel therapeutics warrant exploration. Mesenchymal stem cells and their ability to form organized osseous tissue as

well as their immunomodulatory effects are an attractive candidate for therapy in fracture nonunion.

Hypothesis

Percutaneous introduction of autologous mesenchymal stem cells will show a lower rate on nonunion compared to autologous iliac bone crest grafting used in the treatment of open comminuted tibia fractures treated by locking intramedullary nails. Progression of healing and nonunion will be assessed using modified Radiological Union of Tibia (mRUST) scoring system over the 9 months and a generalized wellness score SF-12 1.

Objectives and specific aims

This study is designed to evaluate the therapeutic use of mesenchymal stem cells in fracture nonunions of the lower extremity long bones. The current gold standard of therapy is iliac crest bone autograft. However, this is associated with significant morbidity to the donor site.[18] By using concentrated bone marrow aspirate, morbidity from the harvest can be reduced. Additionally, assessment of patient-centered outcomes allows for a more holistic comparison of the two treatment paradigms.

The specific aims of this study are as follows.

1) The standard time of nonunion will be assessed at 9 months for a long bone of the lower extremity. Nonunion will be assessed radiographically using a modified Radiological Union of Tibia (mRUST) scoring system over the 9 months' period at each patient recall. Scoring will be in a blinded manner by five independent orthopedic trauma

surgeons with an assessment of the rater intra-class correlation coefficients (ICC) of the reads.

2) Overall progression of healing will be assessed using a generalized wellness score SF-12 Secondary criteria that will be examined include reoperation and general complication rates as well as regain of function.

REVIEW OF THE LITERATURE

Overview

Nonunion is defined as the cessation of all reparative processes of healing without bony union in 9 months since the time of frature.[19] In the United States alone, an estimated 100,000 fractures go on to nonunion each year.[20] The likelihood of fracture nonunion is dependent on both the fracture *per se* and the patient. Risk factors for nonunion include advanced patient age, genetic predisposition, metabolic disease (i.e. diabetes mellitus types 1 and 2), obesity, osteoporosis, rheumatoid arthritis, excessive alcohol use, cigarette smoking, and certain medications like NSAIDs and some antibiotics. Additionally, the type of trauma, fracture, site of injury, and treatment modalities all influence fracture outcomes (please refer to Table 1.).[21]

Nonunion fractures have been shown to have a negative impact on not only patients' physical health, but also their mental well-being.[22] Repeated surgeries, protracted pain secondary to the fracture, and increased medical costs contribute to the burden fracture nonunion patients carry.[3] A study of 237 patients with tibial shaft fracture nonunions compared Short Form (SF)-12 (a questionnaire designed to assess global health) scores against previously published scores for patients with orthopedic conditions, chronic medical conditions, and the general United States population. The results of the study showed physical component summary (PCS) scores (an indicator of global physical health) for the nonunion patients averaged well below the tenth percentile of the United States population (mean nonunion PCS score 27.4 ± 6.7, US mean PCS score 50 ± 10, $p<0.001$). Additionally, average mental component summary (MCS)

scores (an indicator of global mental health) of nonunion patients were found to be below the twenty-fifth percentile of the United States population (mean nonunion MCS score 42.3 ± 7.1, US mean MCS score 50 ± 10, p=0.008).[22] Taken together, this data quantifiably demonstrates the physical and mental toll nonunion has on patients. This data highlights the need for effective therapies in the setting of delayed and nonunion fractures.

Clinical Course of Therapy

Fractures occur when an applied load is greater than the load-bearing capacity of the bone. The greater the applied load (i.e force of the trauma), the more severe the fracture and the greater the risk of nonunion.[21] If the epidermis is breached (i.e. an open fracture) the patient is at an elevated risk of infection, particularly infection of the bone (osteomyelitis). Infection of the fracture site not only perturbs the physiologic mechanisms of fracture repair but also is a significant cause of mortality in patients with open fractures.[23] Thus, current clinical practice dictates empiric intravenous antibiotics and prompt irrigation and debridement of open fractures.[24] Failure to clear infection of a fracture site can contribute towards risk of nonunion, systemic illness, amputation of the affected limb, and even death.[24]

Once an acute fracture patient is hemodynamically stable and acute complications, such as neurovascular injury, are ruled out, the necessity of reduction should be assessed. Reduction is indicated in fractures with significant displacement or angulation. If severely angulated or displaced fractures are allowed to heal without reduction, the healed bone will be deformed and may limit joint motion leading to

disability. The goal of reduction is to bring the fracture edges as close to normal anatomic alignment as possible. Reduction can be performed in an open or closed manner. Closed reduction involves the application of traction to externally manipulate displaced bone fragment into anatomic position. This can be a painful procedure and is typically performed under conscious sedation. Stabilization after closed reduction typically involves casting but may also include percutaneous pinning and splinting. Open reduction may be necessary in fractures with more severe angulation, displacement, and comminution and consists of surgical apposition of the fracture edges under general anesthesia. Open reduction is always stabilized with internal fixation, in which intramedullary nails, screws, and plates bridge the fracture gap, maintaining alignment and providing mechanical stability. If the implants used for mechanical fixation are not stable and significant movement occurs across the fracture gap, hard callus formation is inhibited and risk of nonunion is increased.[25] Thus, care must be taken to ensure sufficient stability across the fracture site. However, surgical choice of internal hardware and technique are fracture and patient dependent.

Postoperatively, patients should be closely monitored for signs of acute compartment syndrome, osteomyelitis, or deep vein thrombosis (venous thromboembolism prophylaxis should begin as soon as is feasible). Delay in recognizing the signs of these complications can lead to death or significant morbidity, including sepsis and amputation. Additionally, adequate pain control is an important aspect in the clinical management of fracture patients. Nonsteroidal antinflammatory drugs (NSAIDs) have been associated with increased risk of nonunion,[21] though a systematic review of the

literature detected no increase in risk among high quality studies.[26] Clinically, patients are typically counseled to avoid NSAID use and take acetaminophen for mild to moderate pain. However, patients with severe fractures often require opioids for sufficient analgesia.

Normal Processes of Fracture Healing

Once proper bony alignment and stabilization has been achieved via interventions discussed above (see Clinical Course of Therapy), the physiologic process of fracture healing takes place in and around the fracture site. While this process is gradual and continuous, it can be conceptually defined in three overlapping stages: (1) the early inflammatory stage, (2) the repair stage, and (3) the late remodeling stage.[27] Immediately following the fracture and resulting damage to adjacent vascular structures, a cascade begins in which transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VGEF), interleukins-1 and -6 (IL-1 and IL-6), bone morphogenetic proteins (BMPs), tumor necrosis factor-α (TNF-α) and other bioactive factors are released promoting angio- and osteogenesis.[28] Compromise of vascular structures around the fracture gap leads to extravasation and hematoma formation. Macrophages, monocytes, and other phagocytic cells clear the surrounding tissue of debris, while activated endogenous MSCs converge on the fracture site via chemotactic mechanisms.[28] The hematoma already has mesenchymal stem cells capable of osteogenic potential, as evidenced by early studies showing transplantation of mouse fracture hematoma into muscle to form new bone by endochondral ossification.[5] Clotting factors transform the hematoma into a fibrinous thrombus which in turn matures into

granulation tissue as angiogenesis leads to capillary invasion of the clot. This weak healing construct has a tensile strength, the force required to pull until it breaks, of about 0.1 MPa (normal bone is 130 MPa).[29] While the timeframe of this process is unique to each patient and fracture, resorption of the 1 to 2 mm of bone at the fracture edges is radiographically apparent 5 to 10 days following the injury.[30]

The second stage of the fracture healing process involves the formation of soft callus, fibrocartilage. Chrondrocytes derived from mesenchymal progenitors produce hyaline cartilage rich in Type 2 collagen, which bridges the fracture gap creating mechanical stability and a template for future ossification.[28] Meanwhile osteoblasts synthesize intramembranous bone formations at the periosteal surfaces. The combination of these structural elements creates a semi-rigid construct with a tensile strength between 4 and 19 MPa, roughly the same tensile strength as rubber (15 MPa).[29] Of note, in fractures with direct apposition of fracture edges hard callus can form without an intervening soft callus template.[28]

The third stage of healing, primary bone formation, entails vascularization and ossification of the soft callus forming hard callus. Osteoblasts synthesize mineralized bone matrix with concomitant vascularization, forming the crude architecture of the hard callus.[28] The hard callus is rigid, with a tensile strength of 130 MPa[29], approximately 32 times as strong as the soft callus construct. While at this point the fracture is clinically healed, physiologic remodeling will continue to take place as the woven bone bridging the fracture site is broken down by osteoclastic activity and replaced with lamellar bone.[29]

The fracture healing process described above typically takes 4 to 6 weeks for simple fractures.[30] Patients are then evaluated for evidence of union, a determination complicated by the fact that there is no diagnostic "gold standard." Several standardized methods of evaluating radiographs for evidence of union have been developed, including the radiographic union scale in tibial (RUST) fractures, discussed below.[31] However, inter-observer correlation is poor and may underestimate healing progress.[32] Therefore, determination of union is a clinical decision based on both radiographic evidence and clinical gestalt[30]

Fracture Healing in Nonunions: Causes and Therapies

Fractures as a result of extreme mechanical force often have radiographic features of comminution (multiple fracture fragments), significant displacement, and angulation. Even with good surgical management, fractures with these features tend towards delayed union, malunion, and nonunion.[25] Delayed union denotes lack of clinical union at 6 months but with radiographic or clinical evidence of continued improvement.[33] Malunion, on the other hand, refers to a healed fracture that is not in anatomic alignment, resulting in a shorter bone length, disruption of joint movement, or physical deformity.[19] If 6-8 months pass and no improvement is seen on serial radiographs and in clinical examinations, the clinician should assess whether the fracture is a delayed union or nonunion. While the FDA classifies nonunion at 9 months, in clinical practice the time at which a fracture is determined as nonunion is not well defined due to the inherent variability in fracture healing times.[19] Delayed union denotes lack of clinical union at 6 months but with radiographic or clinical evidence of continued improvement.[33] Thus,

delayed unions will likely continue to heal, albeit at a protracted rate, whereas nonunions, by definition, have stopped all healing processes and will not result in union without additional intervention.[19]

Nonunion fractures can be broadly classified as either hypertrophic or atrophic.[19] Hypertrophic nonunion fractures are thought to have sufficient vascular supply but lack mechanical stability. Radiographically, hypertrophic nonunion fractures are often horse-show or elephant-foot shaped with decreased callus formation.[19] Atrophic nonunion fractures, on the other hand, are associated with poor vascular supply despite sufficient stabilization. Lack of bony bridging with little callus formation can be observed on radiographs of atrophic nonunion fractures.[19] Due to the biological and mechanical distinctions between these differing nonunion fracture patterns, treatments are different and tailored to address the underlying deficiency.

Hypertrophic nonunion fractures have inadequate mechanical stability and, therefore, treatment aims to increase stability across the fracture site. Stability is achieved through fixation, either internally with nails and screws or externally with casts and immobilization devices. With proper stability, ossification and vascularization of the callus can occur without the need for grafting or osteobiologic intervention.[19]

Atrophic nonunion fractures are more challenging to treat. The current gold standard of therapy involves the application of bone autograft, typically harvested from the iliac crest, into the fracture gap.[6] The graft is then incorporated into the local tissue, stimulating angio- and osteogenesis. Concurrent mechanical stabilization of the fracture is paramount, typically with internal fixation applied during surgical application of the

graft.[19] Choice of bone graft is surgeon-dependent, though not all bone grafts are equally efficacious. Cancellous bone grafts are superior to cortical bone grafts in cellular density, rate of revascularization, and resorption and remodeling time.[6] Bone autograft remains the gold standard in part due to its osteogenic, osteoinductive, and osteoconductive properties.[19] Briefly, osteogenesis entails new bone formation, such as in the reparative process described above. Osteoconduction refers to graft scaffolding for endogenous osteoblasts to use as a framework for osteogenesis. Biologic stimulation of osteoprogenitor cells to differentiate into osteoblasts is known as osteoinduction. These three properties, osteogenesis, osteoconduction, and osteoinduction, represent the three physiologic mechanisms necessary for bone repair to occur.[6] Of the current therapeutic options for atrophic nonunion, bone autograft remains the only one to exhibit the three physiologic properties *per se*.[6] However, iliac crest bone graft involves a high rate of complications and significant morbidity at the donor site, and thus is only undertaken when strictly indicated.[18,34]

Alternative therapeutic options for fracture nonunion include low-intensity pulsed ultrasound (LIPUS), bone marrow aspirate, platelet-rich plasma, recombinant bone morphogenic proteins, demineralized bone matrix, ceramics, allograft bone, and mesenchymal stem cells. While early randomized controlled trials of LIPUS use in nonunions appeared promising,[35] a recent meta-analysis did not show that LIPUS reduces the incidence of fracture nonunion.[36] Given that LIPUS purportedly works via osteoinductive mechanisms,[37] it is perhaps unsurprising that LIPUS therapy alone can be insufficient for recalcitrant nonunions. Similarly, platelet-rich plasma (PRP) works via

osteoinductive mechanisms and can be obtained in an autologous manner via relatively non-invasive peripheral phlebotomy. However, the osteoinductive effect of the concentrated growth factors found in PRP has failed to translate into improved clinical outcomes compared with bone morphogenic proteins.[38] Bone morphogenic proteins (BMPs) are involved in the osteoinduction of endogenous osteoblasts. Commercially available recombinant BMPs have been studied in conjunction with surgical debridement and stabilization,[39,40] but their efficacy cannot be fully assessed because no appropriate control groups were available for comparison in these studies.[6]

While LIPUS, PRP, and BMPs are osteoinductive, they are not osteoconductive. That is to say, application of these therapies may induce osteoblastic activity in endogenous cells, but without a framework with which to conduct this activity, osteogenesis may not take place. Ceramics such as calcium sulfate and hydroxyapatite have been explored as an osteoconductive scaffold in fracture repair. However, while these synthetic "spacers" can be used in conjunction with osteoinductive elements, they are rarely used alone.[6] Demineralized bone matrix (DBM), on the other hand, is both osteoinductive and osteoconductive. By acidifying allograft bone, minerals are chemically removed while the collagen, non-collagenous proteins, and growth factors remain. The resulting DBM can be used to stimulate new bone formation (osteoinduction) as well as provide a collagenous matrix for osteogenesis to take place (osteoconduction).[6] However, DBM still relies on endogenous osteoblasts for osteogenesis.

Allograft bone is very similar to DBM and depending on the preparation can be both osteoconductive and osteoinductive. However, allograft has an inherent potential for graft vs host disease, in which the recipient's immune system rejects the donor material.[41] Bone marrow aspirate, on the other hand, is autologous and therefore will not precipitate an immune response. Composed of hematopoietic and mesenchymal stem cells as well as growth factors, bone marrow aspirate has been shown to be osteogenic, osteoinductive, but not osteoconductive.[6] By centrifuging the bone marrow aspirate, a process that can be done while in the operating room, the stem cells and osteobiologic factors can be concentrated, thus theoretically enhancing their therapeutic effect.[42]

While each of these therapeutic modalities has potential utility in the treatment of nonunion fractures, the unique biology of mesenchymal stem cells makes them an attractive candidate for therapeutic application in this setting.

Brief Overview of Stem Cell Biology

Since the successful cultivation of mouse stem cells in 1981, stem cells have been the focus of extensive scientific research.[43] Stem cells have the infinite capacity to self-renew (replicate) and ability to differentiate into specific cellular lineages, a phenomenon referred to as potency. Some stem cells, such as embryonic stem cells, are pluripotent, or able to differentiate into any cell type in the body.[44] Other stem cells, such as mesenchymal stem cells (MSCs), are multipotent, or able to differentiate into a certain subset of cells, such as bone, cartilage, and fat. The discovery of stem cells' ability to differentiate into a multitude of different specialized cells inspired the search for ways in which stem cells could be used clinically. In pursuit of this search the field of

regenerative medicine was born. Regenerative medicine aims to utilize stem cells to reconstruct tissue injured by disease or trauma.[45] However, nascent stem cell research was plagued with ethical, moral, political, and legal quandaries as this new science brought forth new questions and difficulties.[44]

Research on stem cells arose not from studies of embryonic development, but from careful characterization of teratomas, or abnormal gonadal growths.[44] These tumors are comprised of teeth, skin, hair, sebaceous material, and more adult tissues all arranged in a haphazard conglomerate. Work by Leroy Stevens and Barry Pierce in the 1950s and 1960s demonstrated the existence of a particular cell within teratomas capable of differentiating into multiple adult cell types, now referred to as a multipotent stem cell.[46] Largely thanks to the foundational research on embryonic carcinoma cells derived from teratocarcinomas, the study of mouse embryonic stem cells advanced rapidly. Isolated mouse embryonic stem cells were successfully tested in their ability to form germline chimaeras following insertion into a tetraploid blastocyst.[44] Germline chimerism in combination with gene targeting by homologous recombination opened the door for transgenetic research. For the first time, genes could be selectively altered, or "knocked out," in whole organisms. The development of gene modification in mice was so transformative to the research landscape the 2007 Nobel Prize in Physiology or Medicine was awarded to the team who developed the method.[47]

While embryonic stem cell research continues to be investigated today — 51 "embryonic stem cell" trials are listed on clinicaltrials.gov — more attention is focused on adult stem cell research in clinical therapy with 6,061 "adult stem cell" trials listed on

clinicaltrials.gov.[48,49] The overwhelming emphasis on adult stem cell research may be due to the politicized and inflammatory nature of embryonic stem cells, but it also may be due to the many advantages of using adult stem cells therapeutically.

The hematopoietic stem cell (HSC) was the first adult stem cell described in the literature. This self-renewing cell type resides in the sinusoids of the bone marrow and can differentiate into any hematopoietic cell.[50] Seminal experiments in the 1950s detailed the capacity for self-renewal and multipotency of HSCs. Administering bone marrow cells from healthy mice intravenously to mice with radio-ablated bone marrow demonstrated HSCs have the ability to restore the complete hematopoietic capacity of animals with non-functional bone marrow.[51,52] More than half a century later this *in vivo* serial reconstitution experimental design remains the gold standard for demonstrating capacity for self-renewal, though *in vitro* methods have become commonplace in the literature.[53]

Hematopoietic stem cells were once thought to be the only type of adult stem cells, but we now know of many more stem cell types, including neuronal,[54] intestinal,[55] mesenchymal,[14] satellite,[56] and epidermal.[57] These adult stem cells can be multipotent or unipotent but are not pluripotent. Therapeutic applications of adult stem cells are generally employed in the organ system from which they were isolated. For example, in one study mesenchymal stem cells (MSCs) isolated from dental pulp produced dentin and dental pulp *in vitro*, whereas bone marrow-derived mesenchymal stem cells (BM-MSCs) formed heterotopic bone tissue.[58] Thus, adult stem cells have differentiation tendencies based on their tissue of origin. This has important clinical and experimental implications

when considering where to isolate stem cells from. For example, in orthopedic interventions BM-MSCs are preferred over MSCs originating from other tissue types.[53]

Mesenchymal stem cells (MSCs) are elusive to define partly due to the multitude of names attributable to them. For example, Dr. Arnold Caplan who first coined the term mesenchymal stem cells in 1991, now refers to them as Medicinal Signaling Cells.[14,59] Other labels bestowed on this population of cells include bone marrow stromal cells, perivascular stem cells, mesenchymal stromal cells, multipotent mesenchymal stem cells and postnatal skeletal stem cells.[53,60] The Medical Subject Heading (MeSH term) assigned to this group of cells by Pubmed is "Mesenchymal Stromal Cells," though they were indexed as "Mesenchymal Stem Cells from 2006-2010.[61] Given their capacity for self-renewal and potential for multi-lineage differentiation (i.e. stem-cell like nature) into many mesenchymal cell types (cartilage, bone, adipocytes, fibrous tissue, smooth muscle, cells, and myelosupportive stroma), as well as the historical usage of the term in the literature, this work will use the term mesenchymal stem cell (MSC), though the author acknowledges the validity of other labels.[53]

The first work on mesenchymal stem cells was performed in 1966 by Friedenstein *et al*., who showed that a sub population of cultured bone marrow cells formed avascular heterotopic bone fragments *in vitro*.[62] This seminal experiment demonstrated the existence of a multipotent nonhematopoeietic stem cell within postnatal bone marrow stroma. In other words, this experiment demonstrated the innate osteogenic potential of certain bone marrow cells. Isolation of these osteogenic bone marrow cells from the hematopoietic cells also found in the bone marrow could only be achieved through

culture methods, as cell sorting and immunological techniques were not yet available. Stromal bone marrow cells, or cells part of the physical and functional framework of the bone marrow, adhere to plastic, and thus could be isolated from hematopoietic cells in culture (Friedenstein & Kuralesova 1971). By plating bone marrow stromal cells at a clonal density, cells capable of density-independent growth (an indicator of propensity for growth)[53] could be isolated and soon became known as colony-forming units-fibroblastic (CFU-Fs) (Castro-Malaspine et al. 1980). Roughly 10-20% of these CFU-F colonies are multipotent, as evinced by *in* vivo studies demonstrating heterotopic ossicle formation (complete with bone cells, stroma, adipocytes, and fibroblasts) with CFU-F transplantation in mice.[63] Thus, a clonal subset of bone marrow stromal cells not only has osteogenic potential, but multipotency as well.

Beyond multipotency, stem cells should also demonstrate the capacity for self-renewal. In hematopoietic stem cells (HSCs), self-renewal was proven by using (1) a defined surface phenotype of HSCs, and (2) serial transplantation as an *in vivo* assay.[15] While studies were performed characterizing the immunophenotypic profile of cultured MSCs,[14,64] direct identification of mesenchymal stem cells from *in situ* to *in vitro* eluded all attempts. However, in 2007 Sacchetti *et al.* showed that CD146+ cells matched clonogenic progenitors, pericytes *in situ*, and were able to generate complete organoids, including the hematopoietic microenvironment.[65] The CD146+ pericytes could then be serially transplanted.[66] This data confirms the capacity of MSCs for self-renewal, as well as identifies MSCs as pericytes.

The evidence listed above supports the idea of a self-renewing MSC which resides in a perivascular niche and is capable of differentiating into cartilage, bone, adipocytes, fibrous tissue, smooth muscle cells, and myelosupportive stroma.[53] This evidence is distinct from a substantial portion of the literature espousing the trophic or immunomodulatory properties of mesenchymal stem cells. According to the trophic model of MSC biology, MSCs are pericytes which are capable of differentiating into osteocytes, chondrocytes, myoblasts, stromal cells, fibroblasts, adipocytes, and other connective tissue cell types.[17] However, studies arguing for the capacity of MSCs to differentiate into these varied and diverse lineages utilize a definition of MSCs that does not include validation of the stem-cell nature of the cells used. Instead, these studies are based on a position statement the International Society for Cellular Therapy (ISCT) put forth outlining minimal criteria for defining multipotent mesenchymal stromal cells. This report stated that MSCs are defined by (1) adherence to plastic, (2) specific surface antigen expression, and (3) multipotent differentiation potential.[67] The defining surface antigens are CD105, CD73 and CD90, markers which must be expressed on 95% or more of a MSC population isolated by flow cytometry. Additionally, these cells must lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II, thus excluding cells of hematopoietic lineage. Using this minimal criteria, studies demonstrated the isolation of "MSCs" from brain, spleen, liver, kidney, lung, bone marrow, muscle, thymus, and pancreas – virtually all post-natal organs and tissues.[60] Thus, it became widely accepted that MSCs are pericytes which reside in every tissue. Coupled with the observation that cells with pericyte characteristics respond to local

injury and participate in the wound healing process, MSCs were characterized as trophic mediators of local inflammatory processes.[17]

Confabulation between ISCT-defined MSCs, pericytes, and true MSCs capable of self-renewal as detailed above, led to the study of the therapeutic potential of MSCs in a variety of conditions, ranging from myocardial infarction to inflammatory bowel disease.[68] Industry investment in MSC application with concomitant contribution to the literature distorted the careful characterization of a self-renewing, multipotent mesenchymal stem cell found in the bone marrow to a trophic cultured "drugstore" capable of delivering therapeutic potential in almost any application.[68] Without stringent verification methods of stem cell biology in cultured MSCs, these studies espousing the trophic and immunomodulatory methods of MSCs must be considered with a high degree of skepticism.[15]

Existing Research

The therapeutic application of mesenchymal stem cells (MSCs) in orthopedic conditions has been studied in human clinical trials since 1986. Connolly and Schindell demonstrated that in 100 patients with tibial nonunion application of 100-150 mL of marrow osteoprogenitor cells resulted in better outcomes compared with standard open iliac crest grafting.[69] This pioneering study opened the door for a series of investigations into different preparations of MSCs in varied orthopedic settings.

Garg *et al.* showed that in 20 cases of nonunion of long bones (15 tibia, 3 humerus, and 2 ulna) percutaneous autogenous bone marrow grafting of 15-20 mL of bone marrow resulted in union in 17/20 cases.[70] This study opened the possibility of

nonsurgical resolution of fracture nonunion. A trial in 2005 of 20 cases of tibial nonunion showed that percutaneous autogenous bone marrow grafting (15 mL of bone marrow) resulted in 15/20 union with 4 patients exhibiting no sign of union.[71] This study builds upon the earlier work by Garg *et al.* and proves that percutaneous injection of bone marrow is a simple, safe, inexpensive, and effective therapy in fracture nonunion.[72]However, these studies employed bone marrow aspirate, a mixture of both hematopoietic and mesenchymal stem cells as well as other factors, so the therapeutic effect of MSCs alone cannot be ascertained from this study.

A study in 2005 by Hernigou *et al.* attempted to quantify the therapeutic contribution of MSCs by correlating them to CFU-Fs derived from concentrated bone marrow aspirate.[42] This study found that 53/60 atrophic tibial nonunions inoculated with concentrated bone marrow aspirate achieved union. Beyond demonstrating the therapeutic effect of this intervention, this study also compared computerized tomography scans with the concentration of CFU-Fs in the concentrated bone marrow injected for each patient. They found that there was a positive correlation between the volume of mineralized callus at four months and the number ($p=0.04$) and concentration ($p=0.01$) of CFU-Fs in the graft. However, it should be noted that not all CFU-Fs are clonal populations of MSCs, as only a portion of them can form ectopic bone when transplanted.[53] Additionally, the range of cells applied in this study varied greatly (60-6120 progenitors/cm(3)),[42] highlighting the necessity for a standardized concentration technique.

In 2001, Quarto *et al.* bridged the gap between the laboratory and the operating room and demonstrated that demonstrates that *ex vivo* expanded MSCs can be used with an osteoconductive scaffold to heal large bone defects.[73] Bone marrow cells were expanded in culture, seeded on hydroxapatite ceramic scaffold, and implanted at the lesion sites. Mechanical stability was ensured by external fixation. This small case study found that cultured MSC application was safe and effective in 3 patients with large bone diaphysis defects.[73] It should be noted, however, that while the cultured MSCs in this study were shown to form ectopic bone (i.e. osteogenic capacity), they were not shown to be self-renewing via serial transplantation. Additionally, as this was a small case study, more patients and appropriate controls would be needed to prove the therapeutic effect of MSCs.

Kitoh *et al.* enhanced the osteogenic capacity of culture-expanded bone marrow cells (not specifically MSCs) with the concomitant use of PRP in femoral and tibial lengthening.[74] Bone lengthening, or distraction osteogenesis, entails fixation of a fracture without apposing the fracture ends, allowing the callus to form across the fracture gap thus preserving limb length. The healing index is a widely used parameter employed to quantify the quality and speed of bone formation and is measured in distraction-consolidation time (DCT), or time to bony stability, per cm (DCT/cm). In this study, they found that the healing index of the bone marrow cell and PRP treated bones (n=51) was significantly lower than the control group that received no cell therapy (n=60). However, this study was performed in the pediatric population (average age 15.0 ± 3.21 years) and

thus generalizability to the adult population is questionable. Additionally, this study was retrospective in design, and thus inherently biased.

A multicenter open randomized clinical study in 2009 aimed to assess autologous cultured osteoblast injection compared to no treatment in 64 patients with long bone fractures with poor callus formation six weeks after surgery.[75] They found that osteoblast injection accelerated the healing process and was not associated with specific patient complications. This study emphasizes the utility of osteoprogenitor cells in accelerating fracture healing. However, fracture healing was assessed by callus formation score as determined by two blinded radiologists. As noted above, purely radiographic evaluation of fracture healing without clinical correlation is insufficient in evaluating fracture union.[24]

A 2013 randomized prospective preliminary study demonstrated that patients with distal tibia fractures MSCs and PRP on demineralized bone matrix (DMB) vs standard of care had faster union rates.[12] Twenty-four patients were randomized into either minimally invasive intervention (MII) or control. The intervention involved aspirating iliac crest bone marrow and peripheral blood to yield MSCs and PRP, respectively. The MSCs were isolated via CD105+ immunoselection and combined with PRP on a DMB scaffold and injected under fluoroscopic guidance into the fracture site. The control group underwent watchful waiting. The median time to union was twice as long in the control group (3 months) as compared to the MII group (1.5 months). However, because neither group had complications (i.e. delayed or nonunion), the primary outcome of the study was never truly assessed. That being said, a strength of this study involved the use of biologic

controls. Graft material from each graft was implanted in immunodeficient mice, demonstrating *in vivo* bone formation. Thus, by combining the osteogenic potential of MSCs, the osteoinductive properties of PRP, and the osteoconductive scaffold of DMB, this study effectively replicates the bone healing properties of autograft.

In a similar study, Giannoti *et al.* isolated bone marrow derived MSCs from 8 patients suffering from atrophic pseudoarthrosis (soft callus formation without hard callus ossification) of the upper limb and expanded the cells *ex vivo*, osteo-differentiated them in autologouos-based culture conditions, then embedded the MSCs in autologous fibrin clots. The patients were then evaluated for fracture healing for both short and long term follow-up. The study found that this autologous treatment approach was safe and effective and resulted in successful clinical and functional outcomes for all 8 patients.[13] This study, while small in size, demonstrates not only good clinical outcomes for debilitating pseudoarthrosis, but also good laboratory practice in using autologous culture conditions and agents. However, this study did not have an adequate control group or any blindedness in recruitment and intervention, and must be evaluated with these drawbacks in mind.

More recently, a 2015 retrospective study of 86 diabetic patients with distal tibia and ankle nonunions treated with percutaneous injection of bone marrow mesenchymal stem cells resulted in fewer complications and improved healing rates compared to the control group treated with standard bone iliac crest autograft.[76] Bone marrow was aspirated from the iliac crest and treated by using a cell separator. The number of MSCs present in the bone marrow concentrate was inferred from the number of CFU-Fs. The

study found that of the 86 patients to receive MSC therapy, 70 achieved union (82.1%) compared to 53 of the 86 patients treated with iliac crest bone graft (62.3%). The associated donor site morbidity was also assessed in this study. Both minor and major complications as a result of donor site morbidity were significantly lower ($p=0.01$, $p<0.01$, respectively) in the MSC group compared to the bone iliac crest group. This study is limited by its lengthy recruitment period (1990-2012) and small sample size, though the authors argue the sample size appears sufficient. Additionally, this study examined bone healing in diabetic patients and thus the results may not be generalizable to the larger population. However, this study effectively demonstrates improved healing rates in fracture nonunions and fewer donor site complications in patients treated with MSCs compared to iliac crest bone graft.

Taken together, these studies show that application of MSCs with or without concomitant osteobiologic therapy is safe and effective. However, the method of MSC isolation, utility of *ex vivo* culture expansion, inclusion of osteoconductive scaffold, and route of administration are all important variables for which there is no unified consensus.

METHODS

Study design

This study will be a multicenter non-randomized controlled study of percutaneous autologous mesenchymal stem cells (MSCs) vs autologous iliac crest bone graft in patients with open tibia comminuted fractures treated with a locking intramedullary rod that failed to heal within 9 months.

Study population and sampling

The patients will be recruited over a period of five years from the outpatient orthopedic clinics of Boston Medical Center, Brigham and Women's Hospital, and Massachusetts General Hospital. Without publicly available data relating the volume of fracture nonunion treatment at these institutions, this recruitment period is based on incidence of fractures requiring an inpatient level of care at a similar sized institution[77] and the assumption that 5% of those fractures will go on to nonunion. Thus, the estimated volume of fracture nonunions seen at all three hospitals is 56 per year. Inclusion criteria include: open tibia comminuted fractures treated with a locking intramedullary rod; evidence of fracture nonunion 9 months after fracture; the patient is able to understand the nature of the study; and informed written consent is provided by the patient. Exclusion criteria include: age greater than 75; age less than eighteen; legally dependent; signs of infection; positive serology for HIV; pregnancy or breast-feeding; personal history of cancer; immunosuppression; and surgical intervention within the last 9 months. Fracture nonunion will be determined if at 9 months from the injury no radiographic or clinical signs of healing at the fracture site are evident to the practitioner.

The estimated sample size will be 134 fracture nonunions per arm using sample size calculations that assume an 85% union rate in the MSC arm and a 70% union rate in the iliac crest bone graft arm given 80% power and 5% significance. These rates are based on the 2015 Hernigou *et al.* study with the assumption that a mixed patient population will have slightly superior healing rates compared to a solely diabetic population. Thus, a total of 268 nonunion fractures are needed to power our study and our recruitment period is estimated to accrue 280 patients over five years, accounting for some loss to follow-up.

Treatment

The eligible study population will be informed of the associated risks and benefits of both procedures and the rationale for the investigation will be discussed and informed consent approved by the hospital will be signed. Given that the two interventions detailed herein have different morbidities to the donor sites, it is likely that subjects will discern which treatment they received. Thus, this is not a blinded study. However, the patients will be randomized to one of the two treatment arms using a computer generated model.

The autologous iliac crest bone graft group will undergo standard graft harvest procedure. Under general anesthesia, a 4-5 cm incision will be made just below the anterior iliac wing. The overlying tissue will be resected to the periosteum, at which point a small 2x2 cm area on top of the crest will be stripped using a periosteal elevator. Using a rongeur, a small window, approximately 1.5 x 1.5 cm on top of the crest will be formed. A curette will be used to extract cancellous bone from between the inner and outer tables

and local anesthetic will be applied to the donor site. The cancellous bone graft harvested from the anterior iliac crest can then be packed into the fracture gap using surgeon-specific and case-specific methods.

The percutaneous autologous MSC group will undergo bone marrow aspiration. The procedure detailed herein was developed by the Gerstenfeld laboratory at Boston University School of Medicine. Bone marrow will be aspirated from the anterior iliac crest using a single 5 mm incision. After deep insertion of a beveled Jamshidi needle 6 to 8cm long and 1.5mm in diameter into trabecular bone, marrow will be aspirated into a precoated 30 ml syringe that have been rinsed with a buffer solution containing 400mL of phosphate buffered saline, 25 000 units of heparin and 100 ml of human albumin to avoid clotting. The needle will be moved toward the surface through the same insertion site, with successive aspirations made by turning the needle 90 degrees after each aspiration, and retracting the needle 1 cm after 2 aspirations at 90 degrees to each other. Using this method, only 5 cc of bone marrow will be aspirated from any one site, in order to prevent dilution with peripheral blood in the bone marrow space. The aspirate will then be transferred into a bone marrow collection kit to obtain a final volume of 120 ml of bone marrow aspirate. Nucleated cell and platelet counts will be obtained for every sample pre and post concentration. A final sample of 20 ml is obtained for every centrifugal concentration. All samples are sent for routine bacterial and fungal cultures. Two milliliters of every sample will be assessed by FACS analysis (see below).

With regards to the bone marrow concentrate injection, a 1 cm incision will be made through the skin and fascia at the level of the fracture site. Under fluoroscopic

guidance, a guide wire will be inserted through the cortex of the bone, and into the fracture site. A cannulated drill will be directed over the guide wire to within 2mm of the cortical surface. The guide wire will then be withdrawn and 2 ml of water soluble contrast (Visipaque) will be injected through the drill to confirm the area through which the injected bone marrow will spread. Twenty ml of concentrated bone marrow will then be injected into the fracture site, slowly over a period of approximately 3 minutes. Both incisions are closed with absorbable sutures.

Study variables and measures

The primary outcomes are radiographic and clinical features of union. Radiographic evidence from the time of recruitment and 9 months post-intervention will be evaluated by five independent radiologists in a blinded manner. They will score the results according the modified radiographic union score for tibial (mRUST) fractures scoring system. The mRUST scoring system has demonstrated slightly higher intraclass correlation coefficient (ICC) as compared to the RUST scoring system, indicating greater inter-observer reliability with this tool.[78] The radiographic evidence will be evaluated as either union or nonunion by calculating the mean mRUST score from all five radiologists. A mean mRUST score above 11 will be defined as union and below 11 will be nonunion.

Short-Form 12 (SF-12) is a questionnaire developed from the Medical Outcomes study. The aim of the questionnaire is to measure 8 domains of health-related quality of life. Both physical and mental summary scores can be obtained. Each health domain is scored separately from 0 to 100. This self-administered questionnaire is used widely in

orthopedic studies and has high test repeatability.[79] Clinical evidence as documented in the electronic record at the time of follow up will be evaluated by an independent panel of three orthopedic practitioners not otherwise involved in the study. Clinical evidence will be evaluated as either union or nonunion.

MSC concentration will be determined using FACS analysis of the concentrated bone marrow aspirate following standard cell sorting protocols. MSC populations will be identified as CD29+/CD105+/CD31- and their concentration will be calculated for both before and after concentration.

Recruitment

Patients with fracture nonunion who meet the inclusion and exclusion criteria will be recruited from the outpatient orthopedic clinics of Boston Medical Center, Brigham and Women's Hospital, and Massachusetts General Hospital. Each institution will be responsible for generating a list of candidates who receive orthopedic care at their facility. Presentations will also be given at various conferences and meetings in order to make providers aware of this trial and to bolster recruitment. Candidates will be initially informed of this study by mail one week prior to their 9 month follow up appointment with their orthopedic provider. At the time of the visit, the investigators will meet with the patients to provide further details about the trial including risks and benefits. Individuals who choose to be enrolled will sign a consent form to be registered for the study.

Data collection

Before treatment is initiated, a complete physical exam and medical history is required of each patient. Appropriate case-specific radiographic images of fractures are required. Each patient recall will involve standard radiographic evaluation based on the mRUST standardized scoring method. This scoring will be done by five independent readers and the ICC will examine the results to make sure the scores are unbiased. Review of the electronic medical record for each patient will catalogue demographic data as well as comorbidities such as diabetes, rheumatoid arthritis, and smoking status.

Data analysis

Demographic data such as gender, and ethnicity will be analyzed using descriptive statistics. Additional descriptive statistics will be used to characterize anatomic location of the fracture in the tibia as well as radiographic features such as comminution, displacement, and angulation.

A Pearson's chi squared test will be used to analyze the primary outcome: fracture union 9 months post-intervention. This analysis will be carried out on the radiographic evidence, the clinical evidence, and the combined data. A modified RUST score of 11 will be considered union based on the study performed by Litrentra *et* al.[78] If any discrepancy exists between radiographic and clinical evidence of union, the outcome will be declared as nonunion for the combined analysis. Confounding variables such as smoking status and diabetes will be adjusted for using multiple logistic regression analysis. Furthermore, SF-12 results will be analyzed with respect to intervention using

student's t-test analysis to investigate global health outcomes and surgical management. Confounding variables, as above, will be adjusted for using multiple linear regression.

The association between fluorescence activated cell sorting (FACS) and mRUST scores will be statistically analyzed using the non-parametric Spearman's rank-order correlation analysis.

Timeline and resources

In the summer of 2018, the study proposal will be submitted to the IRB. Upon approval, several weeks of coordination between the orthopedic clinics at Boston Medical Center, Brigham and Women's Hospital, and Massachusetts's General Hospital will take place. Staff will be informed of the study and presentations will be made regarding the biological basis and previous clinical data supporting the treatment. Once recruitment begins, the study is expected to run between five and six years for recruitment, treatment, and follow-up. Data analysis and independent evaluation of the data is expected to take up to 1 year. The total projected timeframe of this study is six to seven years.

Resource requirements for this study include both diagnostic instruments and personnel at each clinical site. A study coordinator will be responsible for the logistic oversight of the study. Radiographic imaging modalities such as X-ray, CT, and MRI will be necessary. Experienced surgical teams for both treatment arms will be necessary as well as experienced interventional radiologists for application of the MSCs. Five independent radiologists will be needed for evaluation of the radiological data. Three independent orthopedic practitioners not otherwise involved in the study will be required for evaluation of clinical outcomes. Access to the electronic medical record will be

necessary to retrieve the clinical and demographic data. Reagents and equipment required for preparation of cells for flow cytometry as well as access to a FACS core facility will be necessary for the evaluation of MSC concentration. A data manager responsible for blinding the radiologic and clinical data as well as a statistician to analyze the data will be needed for study completion.

Institutional Review Board

Prior to the initiation of this study, permission must be obtained from the IRB. The protocol of this study will be submitted for full IRB review to the Boston University Medical Campus IRB and to the corresponding IRBs of the participating institutions. Full IRB approval is necessary given the experimental nature of the intervention and the possibility for morbidity and mortality to any human subjects enrolled within this trial.

CONCLUSION

Discussion

The study detailed herein has inherent limitations. Fracture nonunion lacks a standardized set of replicable criteria, and thus is a determination based on clinical experience. This study attempts to control for this by using blinded evaluators and assessing one single data point: the six-month follow-up. Also, the interventions can vary in technique from surgeon to surgeon. Detailed description of the interventions is intended to create a more uniform technique, but variations between surgeons is to be expected. Additionally, the patients recruited to this study will all be from the Northeastern United States, a population that has been shown to have lower overall levels of Vitamin D,[80] an important factor in bone health. This may influence the generalizability of the results to the broader population.

The proposed study is not a small one, and thus obstacles inherent in the scope of this study are anticipated. Coordination between multiple hospitals and access to electronic records may prove cumbersome. This study hopes to address these obstacles by using the services of a dedicated study coordinator. In addition, patients may be lost to follow up and never return for a 9-month evaluation, particularly if they have healed clinically. While the sample size is adjusted accordingly, follow-up loss may exceed anticipated rates. Explicit emphasis on the importance of re-evaluation should be imparted to each patient enrolled in this study.

The strengths of this study are in its prospective and blinded design, as well as its correlation between clinical and radiographic outcomes with the concentration of MSCs

applied. Additionally, the control group in this study will receive the gold standard of care for fracture nonunion, providing a compelling comparison for this novel therapeutic.

Summary

Fracture nonunion is a debilitating and costly condition. Nonunions may persist despite appropriate surgical and nonsurgical efforts leading to prolonged pain and disability. While autologous iliac crest bone graft remains the gold standard of therapy, this intervention is associated with significant morbidity to the donor site. Mesenchymal stem cells represent a promising osteogenic therapeutic intervention. Percutaneous injection of MSCs is a relatively non-invasive intervention and is associated with less morbidity than iliac crest bone graft harvest.

Clinical and/or public health significance

Patients who suffer from fracture nonunion face chronic pain daily, eroding their physical and mental health. By exploring a less invasive alternative to iliac crest bone grafts, this study aims to show that percutaneous application of MSCs can lead to better outcomes and reduced economic burden. This would effectively translate the biological properties of MSCs into evidence-based clinical practice.

LIST OF JOURNAL ABBREVIATIONS

Acta Med Iran	Acta Medica Iranica
Acta Orthop Scand	Acta Orthopaedica Scandinavica
Ann N Y Acad Sci	Annals of the New York Academy of Sciences
Annu Rev Cell Dev Biol	Annual Review of Cell and Developmental Biology
Arch Orthop Trauma Surg	Archives of Orthopaedic and Trauma Surgery
BMC Musculoskelet Disord	BMC Musculoskeletal Disorders
Bone Jt Res	Bone & Joint Research
Br J Haematol	British Journal of Haematology
Br Med Bull	British Medical Bulletin
Calcif Tissue Int	Calcified Tissue International
Cancer Res	Cancer Research
Clin Lab Med	Clinics in Laboratory Medicine
Clin Orthop	Clinical Orthopaedics
Genes Dev	Genes & Development
Hand Clin	Hand Clinics
Int Orthop	International Orthopaedics
J Biomech	Journal of Biomechanics
J Bone Joint Surg Am	Journal of Bone and Joint Surgery. American Volume
J Bone Joint Surg Br	Journal of Bone and Joint Surgery. British Volume
J Cell Physiol	Journal of Cellular Physiology
J Cell Sci	Journal of Cell Science
J Dent Res	Journal of Dental Research
J Embryol Exp Morphol	Journal of Embryology and Experimental Morphology
J Orthop Res	Journal of Orthopaedic Research
J Orthop Trauma	Journal of Orthopaedic Trauma
J Pediatr Orthop	Journal of Pediatric Orthopedics
J Trauma	Journal of Trauma
JAMA Surg	JAMA Surgery
Med Care	Medical Care
Mol Ther	Molecular Therapy
Musculoskelet Surg	Musculoskeletal Surgery
N Engl J Med	New England Journal of Medicine
Nat Rev Genet	Nature Reviews. Genetics
Nebr Med J	Nebraska Medical Journal
Orthop Surg	Orthopaedic Surgery
Orthop Trauma	Orthopaedicss and Trauma
Sci Transl Med	Science Translational Medicine
Semin Cell Dev Biol	Seminars in Cell & Developmental Biology
Stem Cell Res	Stem Cell Research
Surg Technol Int	Surgical Technology International
Tissue Eng Part A	Tissue Engineering. Part A

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